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(71) Applicant: DOW AGROSCIENCES LLC [US/US]; 9330 Zionsville Road, Indianapolis, IN 46268 (US).

(72) Inventors: PETOLINO, Joseph, F.; 270 Woodstock Court, Zionsville, IN 46077 (US). PAREDDY, Dayakar, R.; 665 Woodbine Drive East, Carmel, IN 46033 (US). HOPKINS, Nicole, L.; 2518 Chaseway Court, Indianapolis, IN 46268 (US). ARMSTRONG, Katherine; 11202 Bridlewood Trail, Zionsville, IN 46077 (US).

(74) Agent: STUART, Donald, R.; Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN 46268 (US).

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(54) Title: WHISKER-MEDIATED TRANSFORMATION OF PLANT CELL AGGREGATES AND PLANT TISSUES AND REGENERATION OF PLANTS THEREOF

(57) Abstract

Plant cell aggregates and plant tissues can be transformed by elongated, needle-like structures called "whiskers". The process comprises the agitation of plant cell aggregates and plant tissues of the plant to be transformed in the presence of DNA and whiskers, whereby DNA uptake and integration thereof is facilitated. The process may be applicable to other plant cell aggregates and plant tissues which have not proven easily transformable by other techniques.

WHISKER-MEDIATED TRANSFORMATION OF PLANT CELL AGGREGATES
AND PLANT TISSUES AND REGENERATION OF PLANTS THEREOF

This invention relates to a method of using

5   elongated, needle-like microfibers or "whiskers" to
transform plant cell aggregates and selected plant
tissues.

Until recently, genetically manipulated plants were
limited almost exclusively to those events created by

10   application of classical breeding methods. Creation of
new plant varieties by breeding was reserved primarily for
the most agronomically important crops, such as corn, due
to the cost and time needed to identify, cross, and stably
fix a gene in the genome, thus creating the desired trait.

15   In comparison, the advent of genetic engineering has
resulted in the introduction of many different
heterologous genes and subsequent traits into diverse
crops including corn, cotton, soybeans, wheat, rice,
sunflowers and canola in a more rapid manner. However,

20   the intergression of a new transgene into elite germplasm
is still quite a laborious task due to the tissue
culturing and back-crossing needed to produce a
commercially viable, elite, line.

Several techniques exist which allow for the

25   introduction, plant regeneration, stable integration, and
expression of foreign recombinant vectors containing
heterologous genes of interest in plant cells. One such
technique involves acceleration of microparticles coated
with genetic material directly into plant cells (U.S.

30   Patents 4,945,050 to Cornell; 5,141,131 to DowElanco; and
5,538,877 and 5,538,880, both to Dekalb). This technique
is commonly referred to as "microparticle bombardment" or
"biolistics". Plants may also be transformed using

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Agrobacterium technology (U.S. Patent 5,177,010 to
University of Toledo, 5,104,310 to Texas A&M, European
Patent Application 0131624B1, European Patent Applications
120516, 159418B1 and 176,112 to Schilperoot, U.S. Patents
5   5,149,645, 5,469,976, 5,464,763 and 4,940,838 and
4,693,976 to Schilperoot, European Patent Applications
116718, 290799, 320500 all to Max Planck, European Patent
Applications 604662,627752 and US Patent 5,591,616 to
Japan Tobacco, European Patent Applications 0267159, and
10   0292435 and U.S. Patent 5,231,019 all to Ciba-Geigy, U.S.
Patents 5,463,174 and 4,762,785 both to Calgene, and U.S.
Patents 5,004,863 and 5,159,135 both to Agracetus).
Another transformation method involves the use of
elongated needle-like microfibers or "whiskers" to
15   transform cell suspension cultures (U.S. Patents 5,302,523
and 5,464,765 both to Zeneca). In addition,
electroporation technology has been used to transform
plant cells from which fertile plants have been obtained
(WO 87/06614 to Boyce Thompson Institute; 5,472,869 and
20   5,384,253 both to Dekalb; 5,679,558, 5,641,664, WO9209696
and WO9321335 to Plant Genetic Systems).

Despite all of the technical achievements, genetic
transformation and routine production of transgenic plants
in a commercially viable, elite, germplasm is still a
25   laborious task. For example, microparticle bombardment,
while capable of being used either on individual cells,
cell aggregates, or plant tissues, requires preparing DNA-
attached gold particles and optimization of an expensive
and not yet widely available, "gun" apparatus. Techniques
30   involving *Agrobacterium* are extremely limited because not
all plant species or varieties within a given species are
susceptible to infection by the bacterium.
Electroporation techniques are not preferred due to the

extreme difficulties and cost typically encountered in routinely making protoplast from different plant species and tissues thereof and the concomitant low viability and low transformation rate associated therewith.

5   As disclosed herein, applicants have invented a method whereby plant cell aggregates and plant tissues from non-elite and elite germplasm can be directly and inexpensively transformed with a recombinant vector containing the gene of choice using whiskers. Applicants'

10   invention is advantageous over currently used methods in that it is simple, quick and easy to use. Furthermore, applicants' invention is superior to that described in the art in that it eliminates the need to establish Type III callus cultures or establish and maintain cell suspension

15   cultures, and can be used with either Type I or Type II callus, thus, is less germplasm limited. This means that applicants' invention, as described herein, can be used to transform elite genotypes directly thus eliminating the problems and time generally associated with gene

20   intergression.

The present invention relates to the production of fertile, transgenic, *Zea mays* plants containing heterologous DNA preferably integrated into the chromosome of said plant and heritable by the progeny thereof.

25   One aspect of the present invention relates to *Zea mays* plants, plant parts, plant cells, plant cell aggregates, and seed derived from transgenic plants containing said heterologous DNA.

The present invention also relates to the production

30   of fertile, transgenic, *Oryza sativa L.* plants containing heterologous DNA preferably integrated into the chromosome of said plant and heritable by the progeny thereof.

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Another aspect of the present invention relates to
Oryza sativa L. plants, plant parts, plant cells, plant
cell aggregates, and seed derived from transgenic plants
containing said heterologous DNA.

5   The present invention also relates to the production
of fertile, transgenic, *Gossypium hirsutum* L. plants
containing heterologous DNA preferably integrated into the
chromosome of said plant and heritable by the progeny
thereof.

10   Another aspect of the present invention relates to
Gossypium hirsutum L. plants, plant parts, plant fibers,
plant cells, plant cell aggregates, and seed derived from
transgenic plants containing said heterologous DNA.

The invention further relates to a process for
15   producing fertile transformed plants from Type I callus,
Type II callus, hypocotyl-derived callus, or cotyledon-
derived callus by whisker-mediated transformation.

The invention yet further relates to a process for
producing fertile transformed plants from meristematic
20   tissue by whisker-mediated transformation.

Another aspect of the invention relates to fertile,
mature maize plants regenerated from Type I or Type II
callus and transgenic seed produced therefrom.

Another aspect of the invention relates to fertile,
25   mature rice plants regenerated from Type I callus and
transgenic seed produced therefrom.

Yet, another aspect of the invention relates to
fertile, mature cotton plants regenerated from hypocotyl-
derived callus or cotyledon-derived callus and transgenic
30   seed and fiber produced therefrom.

In a preferred embodiment, this invention produces
the fertile transgenic plants described herein by means of
whisker-mediated cell perforation and heterologous DNA

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uptake, said whisker-mediated cell perforation being performed on plant cell aggregates and plant tissues, followed by a controlled regimen for selection and production of transformed plant lines.

5   Other aspects, embodiments, advantages, and features of the present invention will become apparent from the following specification.

The present invention relates to methods for production of fertile transgenic plants and seeds of, for

10   example, the species *Zea mays*, *Oryza sativa L.*, *Gossypium hirsutum L.*, and *Brassica napus* by transforming plant cell aggregates and plant tissues of said species with the DNA construct of interest via whisker-mediated transformation. After transformation, transgenic plants are regenerated

15   from said transformed plant cell aggregates and plant tissues and said regenerated plants express the chimeric DNA construct of interest. The transgenic plants produced herein by the methods described include: all species of corn including but not limited to field corn, popcorn,

20   sweet corn, flint corn, dent corn and the like; all species of cotton; and all species of rice.

The following phrases and terms are defined below:

By "antisense" is meant an RNA transcript that comprises sequences complementary to a target RNA and/or

25   mRNA or portions thereof and that blocks the expression of a target gene by interfering with the processing, transport, and/or translation of its primary transcript and/or mRNA. The complementarity may exist with any part of the target RNA, i.e., the 5' non-coding sequence,

30   3'non-coding sequence, introns, or the coding sequence. Antisense RNA is typically a complement (mirror image) of the sense RNA.

By "cDNA" is meant DNA that is complementary to and derived from a mRNA.

By "chimeric DNA construction" is meant a recombinant DNA containing genes or portions thereof from one or more species in either the sense or antisense orientation.

By "constitutive promoter" is meant promoter elements that direct continuous gene expression in all cell types and at all times (i.e., actin, ubiquitin, CaMV 35S, 35T, and the like).

By "cosuppression" is meant the introduction of a foreign gene having substantial homology to an endogenous gene, and in a plant cell causes the reduction in activity of the foreign gene and/or the endogenous gene product. Cosuppression can be sometimes achieved by introducing into said plant cell either the promoter sequence, the 5' and/or 3' ends, introns or the coding region of a gene.

By "developmental specific" promoter is meant promoter elements responsible for gene expression at specific plant developmental stages, such as in early or late embryogenesis and the like.

By "enhancer" is meant nucleotide sequence elements which can stimulate promoter activity such as those from maize streak virus (MSV), alfalfa mosaic virus (AMV), alcohol dehydrogenase intron 1 and the like.

By "expression" as used herein, is meant the transcription of enzymatic nucleic acid molecules, mRNA, and/or the antisense RNA inside a plant cell. Expression of genes also involves transcription of the gene and may or may not involve translation of the mRNA into precursor or mature proteins.

By "foreign" or "heterologous gene" is meant a gene having a DNA sequence that is not normally found in the

host cell, but is introduced by whisker-mediated transformation.

By "gene" is meant to include all genetic material involved in protein expression including chimeric DNA constructions, genes, plant genes and portions thereof.

By "genome" is meant genetic material contained in each cell of an organism and/or virus.

By "inducible promoter" is meant promoter elements which are responsible for expression of genes in response to a specific signal, such as: physical stimuli (heat shock genes); light (RUBP carboxylase); hormone (Em); metabolites, stress and the like.

By "modified plant" is meant a plant wherein the mRNA levels, protein levels or enzyme specific activity of a particular protein have been altered relative to that seen in an unmodified plant. Modification can be achieved by methods such as antisense, cosuppression, or over-expression.

By "plant cell aggregates", "plant cell lines", and "callus cell lines" is meant proliferating masses of tissue composed of a combination of undifferentiated and differentiated cells undergoing *de novo* morphogenesis and formed by placing a piece of plant material (explant) onto a growth-supporting medium under sterile conditions. The terms "plant cell aggregates", "plant cell lines", and "callus cell lines" are meant to include Type I and Type II callus cultures in monocotyledonous plants and hypocotyl- and cotyledon derived cultures in dicotyledonous plants. The terms defined herein are not intended to include either plant cell suspension cultures or Type III callus cultures.

By "plant tissues" is meant organized tissues including but not limited to meristems, embryos, pollen, cotyledons, germ cells, and the like.

By "promoter regulatory element" is meant nucleotide
5 sequence elements within a nucleic acid fragment or gene which controls the expression of that nucleic acid fragment or gene. Promoter sequences provide the recognition for RNA polymerase and other transcriptional factors required for efficient transcription. Promoter
10 regulatory elements from a variety of sources can be used efficiently in plant cells to express sense and antisense gene constructs. Promoter regulatory elements are also meant to include constitutive promoters, tissue-specific promoters, developmental-specific promoters, inducible
15 promoters and the like. Promoter regulatory elements may also include certain enhancer sequence elements that improve transcriptional or translational efficiency.

By "tissue-specific" promoter is meant promoter elements responsible for gene expression in specific cell
20 or tissue types, such as the leaves or seeds (i.e., zein, oleosin, napin, ACP, globulin and the like).

By "transgenic" is meant to include any Type I or Type II callus, hypocotyl- or cotyledon derived callus, tissue, plant parts or plants which contains heterologous
25 DNA or a chimeric gene construct that was introduced into said callus, tissue, plant parts or plants by whiskers and was subsequently transferred to later generations by sexual or asexual cell crosses or cell divisions.

By "whiskers" is meant elongated needle-like bodies
30 capable of being produced from numerous substances as described in "The Condensed Chemical Dictionary, Seventh Edition, Ed. Arthur & Elizabeth Rose, Reinhold Publishing Corp., New York (1966). The invention is not meant to be

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limited to the material from which the whiskers are made
but instead is meant to define a needle-like shaped
structure wherein said whisker is smaller than the cell
for which it is intended to be used in the transformation
5   thereof. It is within the scope of this invention for
whiskers to be shaped in a manner whereby DNA entry into a
cell is facilitated. It is also intended that the scope
of said invention include any material having a needle-
like shape, said needle-like shaped material being able to
10   perforate a plant cell with or without cell walls and thus
facilitate DNA uptake and plant cell transformation. It is
also intended that the scope of this invention not include
microinjection techniques, such as wherein a DNA molecule
is inserted into a cell by passing said DNA through an
15   orifice intrinsic to a needle, said needle being first
inserted into said cell. Preferably, whiskers are metal or
ceramic needle-like bodies, with those most preferred
being made of either silicon carbide or silicon nitride
and being 30x0.5 μm to 10x0.3 μm in size.
20   By "whisker-mediated transformation" is meant the
facilitation of DNA insertion into plant cell aggregates
and/or plant tissues by whiskers and expression of said
DNA in either a transient or stable manner.
          In producing plant cell lines, tissues of interest
25   are aseptically isolated and placed onto solid initiation
medium whereby processes associated with cell
differentiation and specialization occurring in organized
plant cell tissues are disrupted, thus resulting in said
tissues becoming dedifferentiated. Typically, initiation
30   medium is solidified by adding agar or the like because
callus cannot be readily initiated in liquid medium.
Media are typically based on the N6 salts of Chu et al.,
(1978, Proc. Symp. Plant Tissue Culture, Peking Press, p

43-56) being supplemented with sucrose, vitamins, minerals, amino acids, and in some cases, synthetic hormones. However, callus tissues can also proliferate on media derived from the MS salts of Murashige and Skoog, (1962 Physiol. Plant. 15: 473-497). Cultures are generally maintained in a dark, sterile environment at about 28° C.

Typically, plant cell lines are preferably derived from tissues found in juvenile leaf basal regions, immature tassels, hypocotyl tissue, and cotylendonary nodes. For maize and rice, plant cell lines which produce meristematic tissue can be used, with those from zygotic embryo tissue being most preferred. Tissues most preferred for producing said plant cell lines are isolated from developing maize ears 10 to 14 days after pollination and non-germinated rice seed. Hypocotyl and cotyledon-derived tissues from seedlings are most preferred for production of cotton plant cell lines.

After placing said tissues on solid medium, new meristems arise after several days to a few weeks from either the scutellar region, in the case of corn and rice, or from hypocotyl or cotyledonary tissue in the case of cotton. These new meristems produce undifferentiated parenchymatous cell aggregates without the structural order characteristic of the tissue from which they are derived. Plant cell aggregates lack any recognizable overall structure and contain only a limited number of the many different kinds of specialized cell types found in intact, organized plant tissues. Said aggregates have been classified into non-embryogenic and embryogenic depending on their regenerative capacity, mode of reproduction, and tissue morphology (Franz, 1988, Ph.D. Thesis, University of Wageningen, The Netherlands).

10

In corn and rice, non-embryogenic calli are comprised of soft, granular, translucent tissue consisting of elongated, vacuolated cells incapable of plant regeneration. Alternatively, embryogenic, monocotyledonous calli, being capable of somatic embryogenesis and plant regeneration, exist in three distinct morphotypes: Type I; Type II; and Type III.

Type I callus consists of compact, nodular, slow-growing embryogenic callus which proliferates as a mixture of complex tissues exhibiting shoot and/or scutellar-like structures (Phillips et al, 1988, In, Corn and Corn Improvement, pp 345-387). Said callus is characterized by a high degree of cellular differentiation, well developed vascular structures and has been referred to as compact embryogenic callus (US Patent 5,641,664 to Plant Genetic Systems). Essentially all monocotyledonous plants have tissue from which Type I callus can be produced. Plant regeneration in Type I callus normally occurs either through organogenesis by elongation of meristems (Green and Phillips, 1975, Crop Sci., 15:417-421) and/or through somatic embryogenesis from a well defined root-shoot axis (Vasil et al., 1984, Amer. J. Bot. 71:158-161). The origin of regenerated shoots in Type I callus is not always obvious and appears to take place via sub-epidermal meristem formation (Franz and Schel, 1991, Can. J. Bot. 69:26-33).

Type II callus, which is not as common as Type I, consists of soft, friable, embryogenic cells and can only be generated from certain monocotyledonous genotypes (Phillips et al, 1988, In, Corn and Corn Improvement, pp 345-387). It grows rapidly, contains little or no vascular elements and can be described as friable, embryogenic callus (US Patent 5,641,664 to Plant Genetics

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Systems). A distinguishing feature of Type II callus is that it contains numerous globular somatic embryos attached to suspensor-like structures on its surface through which plant regeneration appears to progress in

5   clearly identifiable stages (Franz, 1988, Ph.D. Thesis, University of Wageningen, The Netherlands).

Type III callus has only been most recently described. Said callus is formed only very rarely, is easily dispersed in liquid and does not have any distinct

10   somatic embryos on its surface. It has also been described as "friable, non-mucilaginous" callus (Shillito et al., 1989, Bio/Technology 7:581-587) and consists predominately of undifferentiated tissues capable of regeneration via somatic embryogenesis. Type III callus

15   is considered the ideal tissue for transformation and regeneration because cells thereof easily disassociate and disperse, and thus, readily form suspension cultures. This type of callus is most rare, distinct from Type II callus, and can only be produced by visually selecting and

20   preferentially enriching for it at each sub-culture passage of Type II cultures (WO94/28148; Zeneca).

In cotton, as with other dicotyledonous plants, callus types are typically not defined as Type I, II, or III. Moreover, hypocotyl- and cotyledon-derived callus

25   cultures from cotton have been classified into non-embryogenic and embryogenic depending on morphology and regenerative capacity (Shoemaker et al., 1986 Plant Cell Rep. 3:178-181). Non-embryogenic callus is comprised of a loose, friable mass of cells that does not exhibit a

30   strong cytoplasm staining reaction and cannot be used to readily regenerate plants. However, embryogenic callus appears as a tightly compact, dense cytoplasmic mass of cells capable of plant regeneration via somatic

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embryogenesis. Somatic embryos produced therefrom first appear as globular structures which gradually elongate and then begin to exhibit cotyledonary development.

Of the callus types disclosed herein, Type I and Type II callus cultures derived from monocotyledonous plants are preferred in the production and regeneration of plants. Transgenic maize plants generated via whisker-mediated transformation are most preferably made from either Type I or Type II cultures; whereas, Type I callus cultures are most preferred in the production and regeneration of transgenic rice plants. In addition, cotyledonary node derived and hypocotyl-derived cultures are preferred in the production of transgenic cotton plants, with cultures produced from cotyledon tissue being most preferred.

Shoot tips of plants, including maize, rice, and cotton, contain apical meristems where organ primordia form from apical initial and subepidermal cells (Steeves and Sussex, 1989, Patterns in Plant Development, Cambridge University Press). Meristems can be isolated, placed onto shoot multiplication medium, and induced to produce multiple shoots from which plants can be regenerated (Zhong et al., 1992, Planta 187:483-489). Meristems, either freshly isolated or precultured to initiate shoot multiplication, can serve as recipient tissues for whisker-mediated transformation as taught in the present invention. Shoot tips containing meristematic regions are preferably removed from developing embryos (Lowe et al., 1995, Bio/Technology 13:677-682) or germinating seedlings (Gould et al., 1991, Plant Physiol. 95:426-434), transformed using whiskers with or without an osmotic pretreatment, and placed onto shoot proliferation medium

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containing a selection agent prior to plant regeneration
(Zhong et al., 1996, Plant Physiol. 110:1097-1107).

The heterologous DNA used for transformation herein
may be circular, linear, double-stranded or single-
5   stranded. Generally, said DNA is a recombinant vector
plasmid and contains coding regions therein which serve to
promote expression of the heterologous gene of interest as
well as provide a selectable marker whereby those tissues
containing said gene can be identified. Preferably, these
10   recombinant vectors are capable of stable integration into
the plant genome where selection of transformed plant
lines is made possible by having said selectable marker
expression driven either by constitutive, tissue-specific,
or inducible promoters included therein.

15   One variable present in a heterologous DNA is the
choice of the chimeric gene. Chimeric genes, either in
the sense or antisense orientation, are expressed in plant
cells under control of a constitutive, tissue-specific,
developmental, or inducible promoter and the like.
20   Preference for a particular chimeric gene is at the
discretion of the artisan; however, chimeric genes can be,
but are not limited to, from plants, animals, or bacteria
and the like and can used to express proteins either not
found in a non-transformed cell or found in a transformed
25   cell. Chimeric genes can be also used for, but are not
limited to, up-regulation or down-regulation of an
endogenous gene of interest.

Another variable is the choice of a selectable
marker. Preference for a particular marker is at the
30   discretion of the artisan, but any of the following
selectable markers may be used along with any other gene
not listed herein which could function as a selectable
marker. Such selectable markers include but are not

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limited to aminoglycoside phosphotransferase gene of
transposon Tn5 (Aph II) which encodes resistance to the
antibiotics kanamycin, neomycin and G418, as well as those
genes which encode for resistance or tolerance to
5   glyphosate; hygromycin; methotrexate; phosphinothricin
(bialophos); imidazolinones, sulfonylureas and
triazolopyrimidine herbicides, such as chlorsulfuron;
bromoxynil, dalapon and the like.

In addition to a selectable marker, it may be
10 desirous to use a reporter gene. In some instances a
reporter gene may be used with or without a selectable
marker. Reporter genes are genes which are typically not
present in the recipient organism or tissue and typically
encode for proteins resulting in some phenotypic change or
15 enzymatic property. Examples of such genes are provided
in K. Weising et al. Ann. Rev. Genetics, 22, 421 (1988),
which is incorporated herein by reference. Preferred
reporter genes include the beta-glucuronidase (GUS) of the
uidA locus of *E. coli*, the chloramphenicol acetyl
20 transferase gene from Tn9 of *E. coli*, the green
fluorescent protein from the bioluminescent jellyfish
Aequorea victoria, and the luciferase genes from firefly
Photinus pyralis. An assay for detecting reporter gene
expression may then be performed at a suitable time after
25 said gene has been introduced into recipient cells. A
preferred such assay entails the use of the gene encoding
beta-glucuronidase (GUS) of the uidA locus of *E. coli* as
described by Jefferson et al., (1987 Biochem. Soc. Trans.
15, 17-19) to identify transformed cells.
30 Another variable is a promoter regulatory element.
In addition to plant promoter regulatory elements,
promoter regulatory elements from a variety of sources can
be used efficiently in plant cells to express heterologous

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genes. For example, promoter regulatory elements of
bacterial origin, such as the octopine synthase promoter,
the nopaline synthase promoter, the mannopine synthase
promoter; promoters of viral origin, such as the

5   cauliflower mosaic virus (35S and 19S), 35T (which is a
re-engineered 35S promoter, see PCT/US96/1682; WO 97/13402
published April 17, 1997) and the like may be used. Plant
promoter regulatory elements include but are not limited
to ribulose-1,6-bisphosphate (RUBP) carboxylase small

10   subunit (ssu), beta-conglycinin promoter, phaseolin
promoter, ADH promoter, heat-shock promoters and tissue
specific promoters.

     Other elements such as matrix attachment regions,
scaffold attachment regions, introns, enhancers,

15   polyadenylation sequences and the like may be present and
thus may improve the transcription efficiency or DNA
integration. Such elements may or may not be necessary
for DNA function, although they can provide better
expression or functioning of the DNA by affecting

20   transcription, stability of the mRNA and the like. Such
elements may be included in the DNA as desired to obtain
optimal performance of the transformed DNA in the plant.
Typical elements include but are not limited to Adh-intron
1, the alfalfa mosaic virus coat protein leader sequence,

25   the maize streak virus coat protein leader sequence, as
well as others available to a skilled artisan.

     Constitutive promoter regulatory elements may also be
used thereby directing continuous gene expression in all
cells types and at all times (e.g., actin, ubiquitin,

30   CaMV 35S, and the like). Tissue specific promoter
regulatory elements are responsible for gene expression in
specific cell or tissue types, such as the leaves or seeds

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(e.g., zein, oleosin, napin, ACP, globulin and the like) and may also be used.

Promoter regulatory elements may also be active during a certain stage of the plants' development as well as active in specific plant tissues and organs. Examples of such include but are not limited to pollen-specific, embryo specific, corn silk specific, cotton fiber specific, root specific, seed endosperm specific promoter regulatory elements and the like. Under certain circumstances it may be desirable to use an inducible promoter regulatory element responsible for expression of genes in response to a specific signal, such as: physical stimulus (heat shock genes); light (RUBP carboxylase); hormone (Em); metabolites; and stress. Other desirable transcription and translation elements functional in plants may also be used. Numerous plant-specific gene transfer vectors are known and available to the skilled artisan.

Heterologous DNA can be introduced into regenerable plant cell cultures via whiskers-mediated transformation. While a general description of the process can be found in US Patents 5,302,523 and 5,464,765, both to Zeneca, no protocols have been published to date for whisker-mediated transformation of Type I, Type II, hypocotyl or cotyledon regenerable plant cell lines.

In whisker-mediated transformation, DNA uptake into plant material is facilitated by very small, elongated, needle-like particles comprised of a biologically inert material. When said particles are agitated in the presence of DNA and plant cell lines, one or more of the particles produce small punctures in the regenerable plant cell aggregates thereby allowing said aggregates to uptake the DNA. Cells which have taken up the DNA are considered

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to be transformed. Some transformed cells stably retain
the introduced DNA and express it.

The elongated needle-like particles used in plant
cell transformation are termed "whiskers" and are
5   preferably made of a high density material such as silicon
carbide or silicon nitride; however, any material having a
needle-like structure wherein the size of said structure
is smaller than the cell intended to be transformed is
within the scope of the invention. More preferably,
10 whiskers are made of silicon carbide and are either Silar
SC-9 or Alfa Aesar as described herein.

Before transformation, plant cell lines are
preferably placed onto anosmotic medium and allowed to
incubate thereby enhancing transformation over non-
15 osmotically treated cells. Most preferred is an osmotic
medium having therein about 36.4 g/L sorbitol and about
36.4 g/L mannitol. These tissue are maintained on said
medium for about 4 h before whisker-mediated
transformation. Incubation on medium with osmoticum after
20 transformation at the discretion of the artisan. However,
transformation of non-osmotically treated plant cells
aggregates and tissues is also within the scope of this
invention.

Callus cultures used herein for generation of
25 transgenic plants should generally be about 3 weeks to
about 20 weeks old depending on the culture type.
Typically, callus should be about midway between transfer
periods and thus beyond any lag phase that might be
associated with transfer to a new media or before reaching
30 any stationary phase typically associated with a culture
being on a plate for an extended period of time. However,
plant material can be taken before or after sub-culturing;
therefore, harvest timing is not generally believed to be

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critical to practicing the invention as disclosed herein.
The amount of callus tissue used in each transformation
can vary with amounts of about 100 mg to about 500 mg
preferred, about 100 mg to about 250 mg being more
5   preferred and about 200 mg tissue being most preferred.

For transformation, whiskers are typically placed in
a small container, such as a conical or microfuge tube and
the like, wherein is placed a mixture comprising the DNA
construct of interest, a liquid medium, and callus tissue.
10   The order in which materials are added is not significant
to practicing the invention as disclosed herein.
Thereafter, the container is sealed and agitated. Unlike
particles used in biolistic transformation of plant tissue
(Sanford et al., 1990 Physiol. Plantarum, 79:206-209; and
15   US Patent 5,100,712), whiskers do not require any special
pretreatment with DNA carriers or precipitants prior to
use such as $CaCl_2$, spermidine, sheared salmon sperm DNA and
the like.

Agitation time used in the transformation process can
20   vary and is typically from between about 10 sec to about
160 sec. The amount of whiskers added per transformation
can also vary from between about 1 mg to about 4 mg per
tube. An inverse relationship is observed between the
amount of whiskers added and the agitation time needed to
25   obtain optimal transformation. Therefore, the amount of
whiskers added and the agitation time needed to achieve
transformation is determinable by one having skill in the
art. In addition, the volume of liquid medium added can
vary from about 200 µL to about 1000 µL, with about 200 µL
30   being preferred. Moreover, the amount of heterologous DNA
added can vary from a preferred amount of about 10 µL to
about 100 µL of 1 mg/mL solution. The volume of DNA added
is not as critical of factor to the invention as disclosed

herein as the final DNA concentration. However, preferred
final DNA concentrations are from about 0.03 µg/µL to
about 0.14 µg/µL. The scope of the present invention is
not intended to be limited to said container size, the
5   amount or concentration of heterologous DNA added, the
volume of heterologous DNA added, the amount of the liquid
medium added, the amount of callus material added or the
amount of whiskers added as disclosed herein. The scope
of the invention is also not intended to be limited by the
10   instrumentation used to agitate the mixture or whether
agitation is accomplished by manual or mechanical means.

Once the plant cell lines have been perforated and
the heterologous DNA has entered therein, it is necessary
to identify, propagate, and select those cells which not
15   only contain the heterologous DNA of interest but are also
capable of regeneration. Said cells and plants
regenerated therefrom can be screened for the presence or
absence of the heterologous DNA by various standard
methods including but not limited to assessment of
20   reporter gene expression. Alternatively, transmission of
a selectable marker gene along with or as part of the
heterologous DNA allows those cells containing said DNA to
be identified by use of a selective agent.

Selection of only those cells containing and
25   expressing the heterologous DNA of interest is a critical
step in production of fertile, transgenic plants.
Selection conditions must be chosen in such a manner as to
allow growth of transformed cells while inhibiting growth
of untransformed cells, which initially, are far more
30   abundant. In addition, selection conditions must not be
so severe as to cause transformed cells to lose their
plant regenerability, future viability or fertility. A
skilled artisan can easily determine appropriate

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conditions for selecting transformed cells expressing a particular selectable marker by performing growth inhibition curves. Growth inhibition curves are generated by plotting cell growth versus selective agent

5   concentration. Typically, selective agent concentrations are set at a concentration whereby almost all non-transformed cells are growth inhibited but yet are not killed. Preferred are selective agent concentrations wherein 90-99% of non-transformed cells are growth

10 inhibited but yet not killed. Most preferred are selective agent concentrations wherein 97-99% of non-transformed cells are growth inhibited but yet not killed.

Transformed callus tissues transferred and exposed to selective agents are generally incubated on solid medium

15 supportive of growth. The medium preferred for each type of tissue has been well defined in the art. After initial exposure to selective agents, tissues are transferred periodically to fresh medium while maintaining selective agent concentrations. After transformed cell mass has

20 essentially doubled in size, masses showing the most growth and appearing to be healthy are selected and transferred to fresh medium having selective agent concentrations wherein non-transformed cells will be killed. Repeated selection and transference of growing

25 cells to fresh medium result eventually in a selected group of cells comprised almost exclusively of transformed cells containing the heterologous DNA of interest.

Regeneration, while important to the present invention, may be performed in any conventional manner

30 available to the skilled artisan. If cells have been transformed with selectable marker gene, the selective agent may be incorporated into the regeneration media to further confirm that the regenerated plantlets are

transformed. After subsequent weeks of culturing, regenerated plantlet immune to the selective agent can be transferred to soil and grown to maturity.

Callus and plant derived therefrom can be identified
5 as transformants by phenotypic and/or genotypic analysis. For example, if an enzyme or protein is encoded by the heterologous DNA, enzymatic or immunological assays specific for the particular enzyme or protein can be used. Other gene products may be assayed by using suitable
10 bioassays or chemical assays. Other techniques include analyzing the genomic component of the plant using methods as described by Southern ((1975) J. Mol. Biol., 98:503-517), polymerase chain reaction (PCR) and the like.

Plants regenerated from transformed callus are
15 referred to as the R0 generation or R0 plants. Seed produced by various sexual crosses from plants of this generation are referred to as R1 progeny. R1 seed are then germinated to produce R1 plants. Successful transmission and inheritance of heterologous DNA to R1
20 plants and beyond should be confirmed using the methods described herein.

Generally, the commercial value of transformed corn and progeny thereof will be of greatest value if the heterologous DNA can be incorporated into many different
25 hybrids. This may be achieved by incorporating the heterologous DNA into a large number of parental lines directly as described herein by creating plant cell aggregates of said lines and transforming said lines with whiskers. In addition, this may also be accomplished by
30 crossing initial transgenic fertile plants to normal elite inbred lines and then crossing the progeny thereof back to the normal parent. Progeny from this cross will segregate such that some plants will contain the heterologous DNA of

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interest and some plants will not. Crossing of lines is continued until the original normal parent has been converted to a genetically modified line containing the heterologous DNA of interest and also possessing

5   essentially all attributes associated with that line originally. Corn breeding techniques needed to accomplish elite germplasm lines and inbreds thereof are well known to the skilled artisan.

        Particular embodiments of this invention are further

10 exemplified in the Examples. However, those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

15   EXAMPLE 1

        ESTABLISHMENT OF TYPE I and TYPE II MAIZE CALLUS TISSUE

        For Type I callus initiation, zygotic embryos (1.5 -
3.0 mm) of the genotypes H99, Pa91, and a proprietary line, 139/39 (US Patent 5,306,864), were aseptically

20 isolated, *infra.*, and placed onto Type I medium consisting of N6 macro-nutrients and vitamins (Chu, 1978, Proc. Symp. Plant Tissue Culture, pp. 43-56), B5 micro-nutrients (Gamborg et al., Exp. Cell Res. 50:151-158), 30 g/L sucrose, 2.9 g/L L-proline, 100 mg/L myo-inositol, 100

25 mg/L casein hydrolysate, 37 mg/L sodium salt of Ferrous-
.   ethylenediaaminetetraacetic acid (NaFeEDTA), 8 mg/L dicamba, 0.8 mg/L 2,4-D, 4.9 mg/L $AgNO_3$ and 2.5 g/L GELRITE. Type I callus, initiated from the scutellar region of the original zygotic embryo, consisted of

30 compact embryogenic tissue exhibiting scutellar-like structures and shoot meristems. After several passages of selective sub-culturing at 21-28 day intervals, the

cultures were used for transformation experiments
described herein.

For Type II callus initiation, plants of a hybrid
made by inter-mating two S_3 lines derived from a B73 x A188
5 cross (Armstrong et al., 1991, Maize Genet. Coop. News
Lett., 65:92-93) were grown under standard greenhouse
conditions and self- or sib-pollinated (Petolino and
Genovesi, 1994, The Maize Handbook, pp. 701-704). Ten to
14 days after pollination (DAP), developing ears were
10 removed and surface sterilized with 70% (v/v) ethanol for
2 min followed by soaking in 20% (v/v) commercial bleach
for 0.5 h containing a few drops of LIQUI-NOX, then rinsed
several times with sterile H_2O. Immature embryos (1.5 -
3.0 mm) were then isolated and placed onto initiation
15 medium [N6 basal salts and vitamins (Chu, 1978, Proc.
Symp. Plant Tissue Culture, pp. 43-56), 20 g/L sucrose,
2.9 g/L L-proline, 100 mg/L enzymatic casein hydrolysate
(ECH), 37 mg/L Fe-EDTA, 10 mg/L $AgNO_3$, 1 mg/L 2,4-dichloro-
phenoxyacetic acid (2,4-D), and 2.5 g/L GELRITE
20 (Schweizerhall, South Plainfield, NJ) at pH 5.8]. After
2-3 weeks in the dark at 28° C, Type II callus with
numerous globular and elongated somatic embryos was
obtained. An additional 2-3 selective subcultures were
performed to enrich Type II cultures with desirable
25 morphology as described in Welter et al., 1995, Plant Cell
Rep. 14:725-729. Once Type II morphology was established
(4-6 weeks), callus was transferred to maintenance medium
(initiation medium containing 6 mM L-proline and no $AgNO_3$).
Type II callus was used for transformation after about 16-
30 20 weeks from culture initiation.

EXAMPLE 2

CONSTRUCTION OF THE PLASMID pUGN81-3 AND pDAB418

The maize expression vector, pUGN81 3, containing the
ubiquitin promoter regulatory element driving the β-
5   glucuronidase gene was used as disclosed herein. Plasmid
pUGN81-3 was a 8730 base pairs double stranded plant
transformation vector composed of the following sequences
in clockwise order. Nucleotides 1 to 17 encoded a
polylinker having the sequence AGCTTCCGCG GCTGCAG.

10   Nucleotides 18 to 2003 of pUGN81-3 were the maize
ubiquitin promoter and first intron thereof and were PCR
amplified from genomic DNA of maize genotype B73
(Christensen et al., (1992) Plant Mol. Biol. 18:675-689).
Nucleotides 2004 to 2022 of pUGN81-3 encoded a polylinker

15   having the sequence GGTACCCCCG GGGTCGACC. Nucleotides
2023 to 4154 of pUGN81-3 corresponded to nucleotides 2551
to 4682 of plasmid pBI101 (Clontech, Palo Alto, CA)
followed by a polylinker having the sequence ATCGGGAATT
AAGCTTGCAT GCCTGCAGGC CGGCCTTAAT TAA which corresponded to

20   bases 4155 to 4197 of pUGN81-3. Nucleotides 4198 to 4264
of pUGN81-3 corresponded to: GCGGCCGCTT TAACGCCCGG
GCATTTAAAT GGCGCGCCGC GATCGCTTGC AGATCTGCAT GGGTG.
Nucleotides 4265-4776 of pUGN81-3 comprised the double-
enhanced 35S promoter, with nucleotides 4265 to 4516

25   corresponding to nucleotides 7093 to 7344 of the
Cauliflower Mosaic Virus genome (Franck et al., (1980)
Cell 21:285-294). Nucleotides 4525 to 4776 of pUGN81-3
were a duplication of nucleotides 4265 to 4516 with the
linker CATCGATG comprising nucleotides 4517 to 4524

30   between the duplicated sequence. Nucleotides 4777 to 4871
of pUGN81-3 corresponded to bases 7345 through 7439 of the
Cauliflower Mosaic Virus genome (Franck et al., (1980)
Cell 21:285-294). Nucleotides 4872 to 4891 comprised the

linker GGGGACTCTA GAGGATCCAG. Nucleotides 4892 to 5001 of
pUGN81-3 corresponded to nucleotides 167 to 277 of the
Maize Streak Virus genome with base 187 absent (Mullineaux
et al., (1984) EMBO J. 3:3063-3068). Nucleotides 5002 to
5   5223 corresponded to the modified first intron of the
maize alcohol dehydrogenase gene (Adh1-S) (Dennis et al.,
(1984) Nucleic Acids Res. 12:3983-4000). The
modification resulted in removal of 343 nucleotides (bases
1313 to 1656) with bases 1222 to 1312 (intron 5' end) and
10   nucleotides 1657 to 1775 (intron 3' end) of the maize
Adh1-S gene remaining. Nucleotides 5224 to 5257 of
pUGN81-3 corresponded to Maize Streak Virus (MSV)
nucleotides 279 to 312. Both sections of the Maize Streak
Virus, hereinafter MSV, sequence comprised the
15   untranslated leader of the MSV coat protein V2 gene, and
were interrupted in plasmid pUGN81-3 by the modified Adh1
intron. Nucleotides 5258 to 5814 of plasmid pUGN81-3
corresponded to nucleotides 29 to 585 of the
phosphinotricin acetyl transferase (BAR) gene of
20   *Streptomyces hygroscopicus* (White et al., (1989) Nucleic
Acids Res. 18:1062). To facilitate cloning, nucleotides
34 and 575 of the published sequence were changed from A
and G to G and A, respectively. This sequence served as
the selectable marker. Nucleotides 5815 to 5819 comprised
25   the linker GATCT. Nucleotides 5820 to 6089 of pUGN81-3
corresponded to nucleotides 4414 to 4683 of plasmid pBI101
(Clontech, Palo Alto, CA) followed by the linker sequence
ATCGG. The remaining sequence of pUGN81-3 (nucleotides
6095 to 8730) corresponded to the reverse complement of
30   pUC19 (Yanish-Perron et al., (1985) Gene 33:103-119).

    The maize expression vector, pDAB418, containied the
ubiquitin promoter regulatory element driving the β-
glucuronidase gene was used some expression studies. In

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addition this plasmid carried a second gene which served
as a plant selectable marker. Plasmid pDAB418 was a
10,149 base pairs double stranded plant transformation
vector composed of the following sequences in clockwise
5   order. Nucleotides 1 to 31 had the nucleotide sequence
AATTCATCGA AGCGGCCGCA AGCTTCCGCG G. Nucleotides 32 to
2023 of pDAB418 were the maize ubiquitin (Ubi1) promoter
and first intron, and were PCR amplified from genomic DNA
of maize genotype B73 (Christensen et al., (1992) Plant
10   Mol. Biol. 18:675-689). Nucleotides 2024 to 2042 of
pDAB418 comprised the linker sequence GGTACCCCG
GGGTCGACC. Nucleotides 2043 to 3894 of pDAB418
corresponded to nucleotides 2551 to 4402 of plasmid pBI101
(Clontech, Palo Alto, CA) followed by the linker sequence
15   TGGGGAATTG (bases 3895 to 3904). Nucleotides 3905 to 4174
OF pDAB418 corresponded to 4414 to 4683 of pBI101.
Nucleotides 4175 to 4192 were composed of the linker
sequence ATCGGGAATT AAGCTTGG. Base 4193 through 6184 was
composed of a second copy of the maize ubiquitin promoter
20   and first intron as describe above. This sequence was
followed by the linker sequence GTCGGGGATC TA (6185 to
6196). Nucleotides 6197 to 6753 of plasmid pDAB418
corresponded to nucleotides 29 to 585 of the
phosphinotricin acetyl transferase (BAR) gene of
25   Streptomyces hygroscopicus (White et al., (1989) Nucleic
Acids Res. 18:1062). To facilitate cloning, nucleotides
34 and 575 of the published sequence were changed from A
and G to G to A, respectively. This sequence served as
the selectable marker and was regulated by the maize
30   ubiquitin promoter. Nucleotides 6754 to 6758 were
composed of the linker TAACC. Nucleotides 6759 through
7472 functioned as the 3' polyadenylation sequence and
includes bases 21728 through 22441 from the *Agrobacterium*

tumefaciens octopine Ti plasmid pTi15955 (Barker et al.
(1983) Plant Mol. Biol. 2, 335-350. Sequence 7473 through
7504 was composed of the polylinker GGAATTCATC GATATCTAGA
TGTCGAGCTC GG. The remaining sequence of pDAB418

5 (nucleotides 7505 to 10149) corresponded to the reverse
complement of nucleotides from the plasmid backbone
derived from pUC19 (Yanish-Perron et al., (1985) Gene
33:103-119).

EXAMPLE 3

10 WHISKERS PREPARATION, OPTIMIZATION, AND TYPE I CORN CALLUS
TRANSFORMATION

Type I callus was produced from different inbred
lines. For testing, about 250 mg samples of each Type 1
callus was placed into 2.0 mL microfuge tubes (Brinkman

15 Instuments, Inc., Westbury, NY), 300 mL of Type I medium
consisting of N6 macro-nutrients and vitamins (Chu, 1978,
Proc. Symp. Plant Tissue Culture, pp. 43-56), B5 micro-
nutrients (Gamborg et al., Exp. Ce.. Res. 50:151-158), 30
g/L sucrose, 2.9 g/L L-proline, 100 mg/L myo-inositol, 100

20 mg/L casein hydrolysate, 37 mg/L NaFeEDTA, 8 mg/L dicamba,
0.8 mg/L 2,4-D, 4.9 mg/L $AgNO_3$], 20 µL of DNA solution [1.0
mg/mL pDAB418 in 10 mM Tris, 1 mM EDTA, pH 8.0], and 200
µL of a 40 µg/µL (SC-9) whisker suspension was added.
Each tube was then agitated for 20 sec using a Vortex-

25 Genie 2™ and the callus was transferred back to Type I
medium solidified with 2.5 g/L GELRITE and allowed to
incubate for 16 h. Afterwards, callus was assayed for GUS
expression as described, *infra.*, with results summarized
in Table 1.

30   TABLE 1. GUS expression in Type I callus.

Genotype	GUS Expression Units per Sample
H99	31 ± 26
Pa91	22 ± 8
139/39	3 ± 3

Transient GUS expression in Type I callus following
whisker treatment was observed in all genotypes tested.

EXAMPLE 4

WHISKERS PREPARATION, OPTIMIZATION, AND TYPE II CORN
CALLUS TRANSFORMATION

5   A sterilized suspension of silicon carbide whiskers
was prepared by taking about 40 mg of dry whiskers (Silar
SC-9 from Advanced Composite Materials Corp., Greer, SC;
Alfa Aesar from Johnson-Matthey, Ward Hill, MA) and

10   placing them into a pre-weighed 2.0 mL polypropylene tube.
The tube was then re-weighed to determine the amount of
whiskers added, followed by autoclaving. Immediately
before use, a 40 mg/mL whisker suspension was made by
adding maintenance medium, *supra.*, and vortexing at high

15   speed for 1 min.

In the transformation experiments, either 200 or 500
mg samples of Type II callus from different immature
embryo-derived lines were placed into 17x100 mm culture
tubes (Falcon 2059, Becton Dickinson, Lincoln Park, NJ).

20   Into each was added 500 μL of liquid maintenance medium
[minus GELRITE], 80 μL of DNA solution [0.5 μg/μL pDAB418
in 10 mM Tris, 1 mM EDTA, pH 8.0], and 50 μL of a 40 mg/mL
(SC-9) whisker suspension. Each tube was then agitated
for 20 sec using a Vortex-Genie 2™ (Scientific Industries,

25   Bohemia, NY). Negative control experiments were also
performed. In one case, callus tissue was treated with
whiskers in the absence of DNA. In the other cases,
callus tissue was treated with DNA in the absence of
whiskers. Regardless of the treatment, callus was then

30   transferred back to solid maintenance medium having 2.5
g/L GELRITE and allowed to incubate for 16 h in the dark
at 28° C. Afterwards, callus was placed into GUS assay
solution [0.2 M sodium phosphate pH 8.0, 0.1 mM each of

potassium ferricyanide and potassium ferrocyanide, 1.0 M

sodium EDTA, 0.5 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-

glucuronide, and 0.6% v/v Triton x-100]. GUS expression,

defined as counts of blue sectors per sample, was measured

5   after 48 h of incubation at 37° C in the dark and is

summarized in Table 2.

TABLE 2. GUS expression following whisker-mediated
transformation of callus at a fixed DNA concentration
10   (0.0635 µg/µL Final concentration)

Callus Amount/tube Sample	GUS Expression Units Per
200 mg callus + whiskers + DNA	79 $\pm$ 22
200 mg callus + whiskers - DNA	0
200 mg callus - whiskers + DNA	0
500 mg callus + whiskers + DNA	51 $\pm$ 20
500 mg callus + whiskers - DNA	0
500 mg callus - whiskers + DNA	0

20   GUS expression was only observed when whiskers and DNA

were both agitated in the presence of callus. Slightly

higher transient GUS expression was observed when 200 mg

of callus per tube was used as compared to 500 mg per

tube.

25   To test the effect of different DNA concentrations,

about 200 mg samples of Type II callus from different

immature embryo-derived lines were placed into a 17 x 100

mm culture tube (Falcon 2059, Becton Dickinson, Lincoln

Park, NJ). Into each was added 250 µL of liquid

30   maintenance medium, either 10, 25, or 50 µL of DNA (1.0

µg/µL pUGN81-3 in 10 mM Tris, 1 mM EDTA, pH 8.0), and 50

µL of a 40 mg/mL (SC-9) whisker suspension. Each tube was

then agitated for 20 sec using a Vortex-Genie 2™

(Scientific Industries, Bohemia, NY). The callus was then

35   transferred back to semi-solid maintenance medium and

allowed to incubate for 16 h in the dark at 28° C.

Afterwards, callus was assayed for GUS expression as
previously described with the results shown in Table 3.

TABLE 3. GUS expression following whisker-mediated
transformation of 200 mg callus at varied DNA
concentrations.

Final DNA Concentration Sample	GUS Expression Units Per
0.032 µg/µL	105 ± 73
0.077 µg/µL	86 ± 57
0.143 µg/µL	96 ± 76

As little as 10 µg of DNA per tube (0.032 µg/µL)
containing about 200 mg of callus resulted in transient
GUS expression. Increasing the amount of DNA did not
appear to significantly increase transient GUS expression.

About 500 mg samples of Type II callus representing
different immature embryo-derived lines were prepared as
described herein and placed into 17x100 mm culture tubes
(Falcon 2059, Becton Dickinson, Lincoln Park, NJ). Into
each was added 500 µL of liquid maintenance medium and 50
µL of a DNA solution [0.5 µg/µL pDAB418 in 10 mM Tris, 1
mM EDTA, pH 8.0]. The callus/DNA mixture was then allowed
to incubate for 1 h either at room temperature or on ice.
Callus was incubated in the absence of DNA as a negative
control. Afterwards, 50 µL of a 40 µg/µL (SC-9) whisker
suspension was added and vortexed as described herein.
The callus was then transferred back to maintenance medium
solidified with 2.5 g/L GELRITE and allowed to incubate
for 16 h in the dark at 28° C. Afterwards, the callus was
assayed for GUS expression as described previously.
Results are summarized in Table 4.

TABLE 4. GUS expression following whisker-mediated
transformation of callus pre-incubated with DNA for 1 h.

DNA Incubation Sample	GUS Expression Units Per
DNA + callus + whiskers	70 ± 65
DNA + callus → 1 h(ice) → whiskers	23 ± 37
DNA + callus → 1 h(RT) → whiskers	60 ± 27

Incubation of callus with DNA before whisker agitation
resulted in lower transient GUS expression, especially
when the incubation was at room temperature. Best results
were observed when DNA was added immediately prior to
whisker treatment.

About 200 mg samples of Type II callus representing
different immature embryo-derived lines were either placed
onto osmotic medium [maintenance medium containing 36.4
g/L sorbitol and 36.4 g/L mannitol] and allowed to
incubate for 4 h or maintained on maintenance medium. The
callus was then placed into 17x100 mm culture tubes
(Falcon 2059, Becton Dickinson, Lincoln Park, NJ) and into
each was added 200 μL of either liquid osmotic medium or
liquid maintenance medium, 20 μL of DNA solution [1.0
μg/μL pUGN81-3 in 10 mM Tris, 1 mM EDTA, pH 8.0], and 50
μL of 40 μg/μL (Alfa) whisker suspension. Each tube was
then agitated for 20 sec using a Vortex-Genie 2™, callus
was transferred back to either osmotic medium or
maintenance medium solidified with 2.5 g/L GELRITE and
allowed to incubate for 16 h in the dark at 28° C. Callus
was then tested for GUS expression as described
previously.   Results are summarized in Table 5.

TABLE 5.   GUS expression of callus tissue having been osmotically treated before and/or after whisker-mediated transformation.

Osmotic Treatment	GUS Expression Units Per Sample
None	72 ± 19
Before Only	245 ± 80
After Only	67 ± 37
Before and After	222 ± 83

Transient GUS expression was highest in those cultures which included an osmotic treatment before whisker agitation.

About 200 mg samples of Type II callus representing different cell lines were placed onto osmotic medium and allowed to incubate for 4 h. The callus was then placed into 17x100 mm culture tubes (Falcon 2059, Becton Dickinson, Lincoln Park, NJ) and into each was added 200 µL of liquid osmotic medium [minus GELRITE], 20 µL of DNA solution [1.0 mg/mL pUGN81-3 in 10 mM Tris, 1 mM EDTA, pH 8.0] and either 25, 50, or 100 µL of a 40 µg/µl (Alfa) whisker suspension. Each tube was then agitated for either 20- or 60 sec using a Vortex-Genie 2™. The callus was then transferred back to liquid osmotic medium, allowed to incubate for 16 h in the dark at 28° C, and then assayed for GUS expression as described previously. Results are summarized in Table 6.

TABLE 6.   GUS expression of callus tissue transformed with various amounts of whiskers for 20- and 60 sec.

Whisker Sample Amount seconds	GUS Expression Units Per	
	20 seconds	60
25 µL (1 mg/tube)	61 ± 36	136 ± 80
50 µL (2 mg/tube)	70 ± 46	183 ± 126
100 µL (4 mg/tube)	155 ± 92	175 ± 98

Averaged over all whisker amounts, highest GUS expression
was observed following 60 sec agitation compared to 20
sec. However, a 60 sec agitation appeared to

5   substantially damage the callus. High GUS expression was
also observed when 100 µL of the whisker suspension was
used (4 mg whiskers/tube). Our data indicate that
increased whisker amounts can compensate for agitation
time and visa versa. The most preferred conditions was

10   100 µL of whiskers vortexed for 20 sec.

About 500 mg samples of Type II callus representing
different cell lines were placed onto osmotic medium and
allowed to incubate for 4 h. The callus was then placed
into 17x100 mm culture tubes (Falcon 2059, Becton

15   Dickinson, Lincoln Park, NJ) and into each was added 1000
µL of liquid osmotic medium, 27.7 µL of DNA solution [0.72
µg/µL pUGN81-3 in 10 mM Tris, 1 mM EDTA, pH 8.0], and 200
µL of 40 µg/µL whisker suspension using either Silar SC-9
(Advanced Composite, Greer, SC) or Alfa Aesar (Johnson

20   Matthey, Ward Hill, MA) whiskers. Each tube was then
agitated for 20 sec using a Vortex-Genie 2™ and the callus
was transferred back to osmotic medium solidified with 2.5
g/L GELRITE and allowed to incubate for 16 h at 28° C.
Afterwards, callus was assayed for GUS expression as

25   described previously with results summarized in Table 7.

TABLE 7.   Comparison of whisker type on transformation.

Whisker Type Sample	GUS Expression Units per
Silar SC-9	113 ± 48
Alfa Aesar	51 ± 9

Although both worked, better transient GUS expression was observed using Silar SC-9 whiskers than Alfa Aesar whiskers.

About 500 mg samples of Type II callus representing different cell lines were placed onto osmotic medium and allowed to incubate for 4 h. The callus was then placed into either 17x100 mm culture tubes (Falcon 2059, Becton Dickinson, Lincoln Park, NJ) or 15 mL conical centrifuge tubes (Corning 25319-15, Corning, NY). Into each was added 1000 µL of liquid osmotic medium, 20 µL of DNA solution [1.0 mg/mL pUGN81-3 in 10 mM Tris, 1 mM EDTA, pH 8.0], and 200 µL of 40 µg/µL (Alfa) whisker suspension. Each tube was then agitated for 20 sec using either a Vortex-Genie or a Caulk Vari-Mix II (Estrada Dental, Rancho Cucamonga, CA). Callus was then transferred back to solid osmotic medium, allowed to incubate for 16 h and then assayed for GUS activity (Table 8) as described previously. High levels of transient GUS expression were observed following agitation using both the Vortex and Vari-Mix; however, vortexing appeared to be somewhat better.

TABLE 8. The effect of vessel and agitator type on the transformation of callus tissue with whiskers.

Vessel Type	GUS Expression Units Per Sample	
	Vortex	Vari-Mix
Falcon	418 ± 202	256 ± 261
Corning	395 ± 362	353 ± 271
Agitator Average	407 ± 294	304 ± 270

EXAMPLE 5

PRODUCTION AND REGENERATION OF STABLY TRNSFORMED TRANSGENIC MAIZE PLANTS

About 200 mg samples of Type II callus were placed onto osmotic medium and allowed to incubate for 4 h. The

callus was then placed into 17x100 mm culture tubes
(Falcon 2059, Becton Dickinson, Lincoln Park, NJ) and into
each was added 200 µL of liquid osmotic medium, 20 µL of
DNA solution [1.0 µg/µL pUGN81-3 in 10 mM Tris, 1 mM EDTA,

5   pH 8.0], and 100 µL of 40 µg/µL (Alfa) whisker suspension.
Each tube was then agitated for 20 sec using a Vortex-
Genie 2™, the callus was transferred back to osmotic
medium solidified with 2.5 g/L GELRITE and allowed to
recover for ca. 48 h prior to selection.

10   The whisker-treated callus was placed onto selection
medium [maintenance medium with 30 mg/L Basta™ (Hoechst,
Frankfurt, Germany) and no enzymatic casein hydrolysate or
L-proline] and transferred to fresh selection medium every
four weeks for about 3-4 months. After 10-20 weeks,

15   actively growing colonies were isolated and sub-cultured
onto fresh selection medium every two weeks to bulk-up
callus prior to regeneration.

          For plant regeneration, callus was transferred to
induction medium, *infra.,* and incubated at 28° C, 16h/8h

20   light/dark photoperiod in low light (13 mE/m^2/sec) for one
week followed by 28° C, 16h/8h light dark photoperiod in
high light (40 mE/m^2/sec) for one week provided by cool
white fluorescent lamps. The induction medium was
composed of MS salts and vitamins (Murashige and Skoog,

25   1962, Physiol. Plant. 15:473-497), 30 g/L sucrose, 100
.   mg/L myo-inositol, 5 mg/L benzyl amino purine, 0.025 mg/L
2,4-D, 2.5 g/L GELRITE and adjusted to pH 5.7. Following
this two-week induction period, callus was transferred to
regeneration medium and incubated in high light (40

30   mE/m^2/sec) at 28° C. The regeneration medium was composed
of MS salts and vitamins, 30 g/L sucrose, and 2.5 g/L
GELRITE adjusted to pH 5.7. The callus was sub-cultured

to fresh regeneration medium about every two weeks until plantlets appeared. Both induction and regeneration medium contained 30 mg/L Basta™. Plantlets were transferred to 10 cm pots containing approximately 0.1 kg of dry Metro-

5 Mix 360 (The Scotts Co, Marysville, OH), placed in a greenhouse, moistened thoroughly, and covered with clear plastic cups for 2-4 days. At the 3-5 leaf stage, plants were transplanted to 5-gallon pots containing soil and grown to maturity.

10   EXAMPLE 6

SOUTHERN ANALYSIS OF TRANSFORMED CALLUS AND PLANT TISSUES

BASTA resistant lines were characterized by Southern analysis to confirm the presence of the transgene using a DNA probe specific for the coding region of the gene of

15 interest. DNA from both callus and leaf material was analyzed.

For callus, the material was soaked in distilled water for 30 min. and transferred to a new petri dish prior to lyophilization. Leaf material from plants was

20 harvested at the 6-8 leaf stage. Genomic DNA was prepared from lyophilized tissue as described by Saghai-Maroof et. al. ((1984) Proceed. Nat. Acad. Sci. USA 81:8014-8018). Eight μg of each DNA was digested with the restriction enzyme(s) specific for each plasmid construct using

25 conditions suggested by the manufacturer (Bethesda Research, Gaithersburg, MD) and separated by electrophoresis on a 0.8% agarose gel. The DNA was then blotted onto nylon membranes as described by Southern ((1975) J. Mol. Biol., 98:503-517). A 1.9 kb DNA probe

30 specific for the GUS coding region was prepared using an Oligolabelling Kit (Pharmacia LKB, Piscataway, NJ) with 50 mCi of [a-^{32}P]dCTP (Amersham Life Science, Arlington Heights, IL). The radioactive probe was then hybridized to

the genomic DNA on the blots in 45 mL of minimal

hybridization buffer [10% polyethylene glycol, 7% sodium

dodecyl sulfate (SDS), 0.6x SSC where 1X SSC is 0.15 M

NaCl, 0.015 M sodium citrate, pH 7.0, 10 mM sodium

5   phosphate, 5 mM EDTA and 100 μg/mL denatured salmon sperm

DNA] overnight at 60° C. After hybridization, blots were

washed at 60° C in 0.25X SSC and 0.2% SDS for 45 min.,

blotted dry and exposed to XAR-5 film (Kodak, Rochester,

NY) overnight on two intensifying screens (DuPont, Newark,

10 DE).

Plants regenerated from transgenic callus lines were

tested via Southern analysis and all were found to have

the 4.2 kb hybridization product. The hybridizing product

was expected to contain the ubiquitin-1 promoter/intron,

15 the GUS coding sequence, and the nos 3' untranslated

region. Individual plants regenerated from a given

culture all displayed identical hybridization patterns.

EXAMPLE 7

ESTABLISHMENT OF EMBRYOGENIC TYPE I RICE CALLUS
20   TISSUE

For initiation of rice embryogenic Type I callus

cultures, mature seeds of a *Oryza sativa L. cv. Japonica*,

Taipei 309, were dehusked and surface-sterilized in 70%

(v/v) ethanol for 2-5 min followed by a 30-45 min soak in

25 50% (v/v) commercial bleach containing a few drops of

LIQUI-NOX (Alconox, Inc., New York, NY). The seeds were

then rinsed 3 times in sterile H_2O and placed on filter

paper before being transferred to induction medium [N6

macro elements (Chu, 1978, Proc. Symp. Plant Tissue

30 Culture, Peking Press, p 43-56), B5 micro elements and

vitamins (Gamborg et al., 1968, Exp. Cell Res. 50:151-

158), 300 mg/L casein hydrolysate, 500 mg/L L-proline, 500

mg/L L-glutamine, 30 g/L sucrose, 2 mg/L 2,4-D, and 2.5

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g/L GELRITE, pH 5.8]. The seeds were cultured on
induction medium and incubated in the dark at 28° C for 3
weeks. Afterwards, emerging primary callus induced from
the scutellar region was transferred to fresh induction
5   medium for further maintenance. In general, embryonic
callus was selected based on its morphology after each
subculture. The general morphology of rice Type I
embryogenic callus emerged as hard, compact, nodular
structures appearing, and in some cases not appearing,
10   embryo-like. This material was termed Type I.

EXAMPLE 8

WHISKERS PREPARATION, OPTIMIZATION, AND RICE TYPE I CALLUS TRANSFORMATION

Immediately prior to DNA delivery, a sterile 50 µg/µL
15   suspension of silicon carbide whiskers (Silar SC-9) was
prepared in water as described previously. About 125-500
mg of Type I rice embryogenic callus being less than 4
months-old was selected and transferred into either a 15
mL Corning conical centrifuge tube or a 1.5 mL microfuge
20   tube to which about 250 µL of liquid initiation medium
(initiation medium without GELRITE) had been added. Then,
about 10-160 µL of a 50 µg/µL whisker solution was added
along with 25 µL of 1 µg/µL pDAB418 DNA solution
immediately prior to vortexing for 15-120 sec with Vortex
25   Genie 2™ at the highest speed possible. Afterwards, the
callus was transferred to initiation medium with and
without high osmoticum [0.2 M mannitol and 0.2 M sorbitol;
Vain et al., 1993, Plant Cell Rep. 12:84-88] and incubated
in the dark at 28° C for about 24 h before being examined
30   for GUS activity.

Gus histochemical assays were conducted according to
Jefferson et al., (1987, EMBO J 6: 3901-3907) and as
described here. Tissues were placed in 24-well microtiter

plates (Corning, New York, NY) containing 500 μL of assay
buffer per well. The assay buffer consisted of 0.2 M
sodium phosphate (pH 8.0), 0.1 mM potassium ferricyanide,
0.1 mM potassium ferrocyanide, 1.0 M sodium EDTA, 1.9 mM
5   5-bromo-4-chloro-3-indolyl-β-D-glucuronide, and 0.06%
triton X-100. The plates were incubated in the dark for
1-2 days at 37° C before microscopically examining the
tissue for expression. Silicon carbide whisker-mediated
DNA delivery was measured in terms of GUS transient
10   expression, i.e., GUS expression units (blue spots) per
sample.

        Many different transformation parameters were tested
to determine the optimum conditions needed for
transformation. Some of the parameters tested included:
15   vortexing time, amount of the 50 μg/μL whisker solution
added, treatment with high osmoticum before and/or after
whisker treatment, amount of tissue used per
transformation, and vessel size. In all of these
experiments, the protocols described herein were
20   essentially the same unless otherwise mentioned.

        The effect of vortexing time (15-60 sec) was studied
for DNA delivery into rice embryogenic Type I callus. In
this experiment, callus was treated with high osmoticum
for about 4 h prior to transformation with whiskers and
25   for about 16 h following whisker treatment. Callus was
transformed by placing 250 mg of callus into a 15 mL
Corning conical centrifuge tube, adding 1000 μL of liquid
initiation medium, 25 μL of a 1 μg/μL DNA solution
(pDAB418), and 100 μL of a 50 μg/μL whisker solution.
30   Multiple sample sets were vortexed for 15-, 30-, 60- and
120 sec and the results were quantitated as shown in Table
9.

Table 9.   The effect of vortexing time on the
transformation of rice Type I embryogenic callus.

Vortexing Time (Sec)	Gus Expression Units per Sample
15	113.5
30	107.5
60	90.5
120	72.5

As can be seen, DNA delivery and callus transformation was possible when vortexing for as little as 15 sec despite the hard and compact morphology of rice Type I embryogenic callus.

The effect of varying the amount of whiskers added was examined while maintaining a constant volume (1000 µL) of liquid initiation medium. Callus samples were treated with high osmoticum before and after whisker-mediated transformation. About 250 mg of callus tissue was selected and transferred to a 15 mL Corning conical centrifuge tube to which 1,000 µL of liquid initiation medium lacking GELRITE was added. Different amounts of a 50 µg/µL whisker solution (10, 20, 40, 80, and 160 µL) along with 25 µL of a 1 µg/µL DNA solution (pDAB418) were added prior to vortexing with Vortex Genie 2™ for 60 sec. Samples were then transferred back onto the initiation medium with high osmoticum overnight before histochemical GUS assays were performed. The results are described in Table 10. GUS expression was found to correlate with an increasing abundance of whiskers added.

Table 10.   The effect of increasing the amount of whiskers
while maintaining a constant volume of initiation medium.

Whisker Solution Added (µL)	Gus Expression Units/Sample
10	2
20	1
40	9
80	6
160	6.5

The effect of high osmoticum on transformation
efficiency was studied with the results summarized in

5   Table 11.   In this experiment, callus tissue was subjected
to either no high osmoticum treatment or high osmoticum
treatment after whisker-mediated transformation.   About
250 mg of callus tissue was transferred to 1.5 mL
microfuge tube.   About 250 µL of liquid initiation medium

10   was added followed by 125 µL of 50 µg/µL whisker solution
and 25 µL of a 1 µg/µL DNA solution (pDAB418).   The
samples were vortexed for 60 sec and transferred back onto
the initiation medium overnight with or without high
osmoticum before GUS assays were conducted.

15   Table 11.   The effect of osmoticum treatment after
vortexing and vortexing time on transformation and
transgene expression of rice Type I embryogenic callus
tissue.

Osmoticum After Transformation	mg Tissue	Vortex Sec.	GUS Expression Units Per Sample
+	250	30	48
+	250	60	44
+	500	30	37
+	500	60	44
-	250	30	87
-	250	60	107
-	500	30	96
-	500	60	120

20   Callus tissue with no high osmoticum treatment after
whisker transformation resulted in higher expression
levels compared to that where high osmoticum treatment
occurred.

The amount of callus tissue added and vessel size
(1.5 mL microfuge tube vs. 2.0 mL microfuge tube) used
were studied. In this experiment, 125 or 250 mg of callus
tissue were used while maintaining a constant volume of
5   liquid initiation medium, i.e., 250 µL. In all cases, 125
µL of a 50 µg/µL whisker solution along with 25 µL of a 1
µg/µL DNA solution (pDAB418) were added prior to vortexing
with Vortex Genie 2™ for 60 sec. Following
transformation, callus samples were incubated on the
10   initiation medium with high osmoticum overnight before GUS
assays were conducted. The results are summarized in
Table 12.

Table 12. The effect of vessel size and the amount of
15   tissue added on the whiskers-mediated transformation of
rice Type 1 callus tissue.

mg Tissue	Vessel Size (mL)	Gus Expression Units/Sample
125	1.5	8
125	2.0	6
250	1.5	24
250	2.0	9.5

The use of 1.5 mL microfuge tube and 250 mg callus tissue
per tube resulted in highest transient expression.
20

EXAMPLE 9

PLASMID DESCRIPTION FOR pUbiHyg

The rice expression vector, pUbiHyg, contained the
maize ubiquitin promoter and first intron from the
25   ubiquitin gene (Ubi1) regulatory element driving the
hygromycin B phosphotransferase (resistance) gene as
described by Gritz and Davies, (1983) Gene 25:179-188.
Plasmid pUbiHyg was a 5991 base pairs double stranded
plant transformation vector composed of the following
30   sequences in clockwise order. Nucleotides 1 to 42 had the

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sequence AAGCTTGCAT GCCTGCAGAT CTGCGGCCGC AAGCTTCCGC GG.
Nucleotides 43 through 2034 of pUbiHyg were the maize
ubiquitin promoter and first intron, and were PCR
amplified from genomic DNA of maize genotype B73

5   (Christensen et al., (1992) Plant Mol. Biol. 18:675-689)
Nucleotides 2035 to 2052 of pUbiHyg had the sequence
GGTACCCCCG GGTAGACC. Nucleotides 2053 through 3078 of
pUbiHyg corresponded to nucleotides 211 through 1236 of
the the hygromycin B phosphotransferase (resistance) gene

10   sequence (accession number K01193), with bases 2056 and
2057 of pUbiHyg modified from AA to GT to facilitate
future cloning. Bases 3079 through 3097 were composed of
the linker TAAGAGCTCG AATTTCCCC. Bases 3098 through 3351
corresponded to nucleotides 4430 to 4683 of plasmid pBI101

15   (Clontech, Palo Alto, CA). The remaining sequence of
pUbiHyg (nucleotides 3352 to 5991) corresponded to
nucleotides from the plasmid backbone, (Yanish-Perron et
al., (1985) Gene 33:103-119).

EXAMPLE 10

20   PRODUCTION OF STABLY TRANSFORMED RICE TYPE I CALLUS TISSUE
AND REGENERATION OF TRANSGENIC PLANTS
        For production of stable rice transgenic plants, rice
Type I callus tissue was produced as described herein and
subjected to whisker-mediated transformation. Typically,

25   250 mg of rice Type I callus, with or without high
osmoticum treatment for four hours, was added to a 1.5
microfuge tube. Also added were 250 µL of liquid
initiation medium, 125 µL of a 50 µg/µL whisker solution,
and 25 µL of a 1 µg/µL DNA solution (pDAB 305 and UbiHyg

30   in 1:1 ratio). Vortexing was carried out for 60 sec as
described previously to ensure DNA delivery. Following
whisker-mediated DNA delivery, callus was transferred to
initiation medium, as described previously, with high

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osmoticum for 24 h before transfer to selection medium.
Selection medium consisted of initiation medium with 30
mg/L hygromycin (CalBiochem-Novabiochem Corporation, La
Jolla, CA). After 2 weeks, cultures were transferred to

5   fresh selection medium having 50mg/L hygromycin (Li et
al., 1993, Plant Cell Rep. 12: 250-255). Placing cultures
on selection medium resulted in the formation of compact,
white-yellow, embryogenic callus cultures after 30-45
days. These cultures were then transferred to pre-

10   regeneration (PR) medium having 50 mg/L hygromycin and
maintained in the dark at 28° C. PR medium consisted of
initiation medium with 2 mg/L benzyl aminopurine (BAP), 1
mg/L naphthalene acetic acid (NAA), and 5 mg/L abscisic
acid (ABA). After 2 weeks, cultures were then transferred

15   to regeneration (RN) medium wherein RN medium was
initiation medium with 3 mg/L BAP, and 0.5 mg/L NAA.
Cultures on RN medium were incubated for at 28° C under
high fluorescent lights (325-ft-candles) until plantlets
emerged. When the plantlet shoots reached about 2 cm,

20   they were transferred to Magenta GA7 boxes (Magenta Corp.,
Chicago, IL) containing medium consisting of MS macro and
micro nutrients along with B5 vitamins (Gamborg et al.,
1968, Exp. Cell Res. 50:151-158) which had been diluted
1:1 with water, 10 g/L sucrose, 0.05 mg/L NAA, 50 mg/L

25   hygromycin, 2.5 g/L GELRITE, and adjusted to pH 5.8. When
plantlets were established with well-developed root
systems, they were transferred to soil/metromix 360 (1:1)
and raised in the greenhouse (29°/24° C day/night cycle,
50-60% humidity, 12 h photoperiod) until maturity.

30   Southern analysis of these plants revealed the presence of
the hygromycin gene indicating that they were indeed
transformed. These plants were successfully grown to
maturity in the greenhouse.

EXAMPLE 11

PLASMID DESCRIPTION FOR pSMGN179-3

The expression vector, pSMGN179-3, contained a
modified derivative of the chimeric regulatory regions

5   from the *Agrobacterium tumefaciens* opine synthase genes
described by Gelvin and Hauptmann, (1995) Patent WO
95/14098 driving the β-glucuronidase gene. Plasmid
pSMGN179-3 was a 5541 base pairs double stranded plant
transformation vector composed of the following sequences

10   in clockwise order: nucleotides 1 to 16 had the multiple
cloning sequence from the plasmid backbone, pUC19 (Yanish-
Perron et al., (1985) Gene 33:103-119) AATTCGAGCT
CGGTAC. Nucleotides 17 to 57 of pSMGN179-3 were composed
of linker fragment having the sequence CGGGCCCCCC

15   CTCGAGGTCG ACGGTATCGA TAAGCTTGAT C. Bases 58 through 277
of pSMGN179-3 corresponded to the reverse complement of
bases 13774 through 13993 of *Agrobacterium tumefaciens* Ti
plasmid pTi15955 T-DNA (Barker et Gal. (1983) Plant Mol.
Biol. 2:335-350). These bases corresponded to upstream

20   activating sequences from the octopine synthase (ocs)
gene. Bases 278 through 304 corresponded to linker DNA
with the sequence CGAATTCCAA GCTTGGGCTG CAGGTCA. Bases
305 through 350 corresponded to the reverse complement of
bases 21475 through 21520 of *Agrobacterium tumefaciens* Ti

25   plasmid pTi15955 T-DNA (Barker et Gal. (1983) Plant Mol.
Biol. 2:335-350). Bases 351 through 737 of pSMGN179-3
were composed of the reverse complement of bases 20128
through 20514 from *Agrobacterium* b-glucuronidase gene
tumefaciens Ti plasmid pTi15955 T-DNA (Barker et Gal.

30   (1983) Plant Mol. Biol. 2:335-350). The sequence of
pSMGN179-3 from 305 through 737 included the ΔMAS promoter
described by Gelvin and Haupman, (1995) Patent WO
95/14098. Bases 738 through 763 of pSMGN179-3 contain

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linker sequence CTCTAGAACT AGTGGATCCG TCGACC. Bases 58

through 763 of pSMGN179-3 comprised the promoter and

untranslated leader regulatory fusion as given in SEQ ID

NO 1. Nucleotides 764 to 2615 of pSMGN179-3 corresponded

5   to nucleotides 2551 to 4402 of plasmid pBI101 which

encoded the β-glucuronidase gene (Clontech, Palo Alto,

CA)(Jefferson et al., (1986) Plant Mol. Biol. Rep.

83(22):8447-8451). Bases 767 through 769 were modified

from TTA to GTC. The β-glucuronidase gene was followed by

10   the polylinker sequence TGGGGAATTG, corresponding to bases

2616 to 2625 of pSMGN179-3. Bases 2626 through 2895

corresponded to 4414 to 4683 of pBI101 which contained the

sequence from the nopaline synthase 3' untranslated

regions (Clontech, Palo Alto, CA)(Jefferson et al., (1986)

15   Plant Mol. Biol. Rep. 83(22):8447-8451). The remaining

sequence of pSMGN179-3 (nucleotides 4684 to 5541)

corresponded the reverse complement of nucleotides from

the plasmid backbone which was derived from pUC19 (Yanish-

Perron et al., (1985) Gene 33:103-119).

20   EXAMPLE 12

TRANSFORMATION OF EMBRYOGENIC COTTON CALLUS

Approximately 250 mg of cotton callus from different

seedling-derived lines were placed onto osmotic medium

[maintenance medium plus 36.4 g/L sorbitol and 36.4 g/L

25   mannitol] and incubated for 4 h in the dark. The callus

was then placed into a 17 x 100 mm culture tube (Falcon

2059, Becton Dickinson, Lincoln Park, NJ) into which was

added 250 μL of liquid osmotic medium [minus GELRITE], 20

μL of DNA (1.0 μg/μL pSMGN179-3 in 10 mM Tris, 1 mM EDTA,

30   pH 8.0), and 50 μL of a 40 mg/mL (Alfa) whisker

suspension. Each tube was then agitated for either 40,

80, or 160 sec using a Vortex-Genie 2™ (Scientific

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Industries, Bohemia, NY). The callus was then transferred

back to osmotic medium solidified with 2.5 g/L GELRITE and

allowed to incubate at 28° C in the dark for 16 h.

Afterwards, it was assayed for GUS expression as

5   previously described with the results shown in Table 13.

TABLE 13. GUS expression following whisker-mediated
transformation cotton callus using different agitation
times.

Agitation Time Sample	GUS Expression Units Per
40 sec	41 ± 21
80 sec	59 ± 38
160 sec	37 ± 28

GUS expression was observed in whisker-treated cotton

callus. Increasing the agitation time from 40 to 160 sec

did not appear to significantly increase expression.

EXAMPLE 13

20   PRODUCTION OF PLASMID pDAB 305

Plasmid pDAB305 was a 5800 bp plasmid that harbored a

promoter containing a tandem copy of the Cauliflower

Mosaic Virus 35S enhancer (35S), a deleted version of the

Adh1 intron 1, and the untranslated leader from the Maize

25   Streak Mosaic Virus Coat Protein fused to the β-

glucuronidase gene, which was then followed by the *nos*

3'UTR. It was made as follows:

The starting material was plasmid pUC13/35S(-343) as

described by Odell *et al.* (1985 Nature 313:810-812). This

30   plasmid comprised, starting at the 3' end of the Sma I

site of pUC13 (Messing, 1983 *in* Methods in Enyzmology, Wu,

R. Ed. 101:20-78), and reading on the strand contiguous to

the noncoding strand of the *lacZ* gene of pUC13,

nucleotides 6495 to 6972 of CaMV, followed by the linker

35   sequence CATCGATG (which encodes a Cla I recognition

site), followed by CaMV nucleotides 7089 to 7443, followed

by the linker sequence CAAGCTTG, the latter sequence
comprising the recognition sequence for Hind III, which
was then followed by the remainder of the pUC13 plasmid
DNA.

5   The plasmid pUC13/35S(-343) DNA was digested with Cla
I and Nco I, the 3429 base pair (bp) large fragment was
separated from the 66 bp small fragment by agarose gel
electrophoresis, and then purified by standard methods.
The DNA was digested with Cla I, and the protruding ends

10   were made flush by treatment with T4 DNA polymerase. The
blunt-ended DNA was then ligated to synthetic
oligonucleotide linkers having the sequence CCCATGGG,
which included an Nco I recognition site. The ligation
reaction was transformed into competent *E. coli* cells, and

15   a transformant was identified that contained a plasmid
(named pOO#1) that had an Nco I site positioned at the
former Cla I site. DNA of pOO#1 was digested with Nco I
and the compatible ends of the large fragment were
religated, resulting in the deletion of 70 bp from pOO#1,

20   to generate intermediate plasmid pOO#1 NcoÆ.

          The plasmid pOO#1 NcoÆ DNA was digested with EcoR V,
and the blunt ends were ligated to Cla I linkers having
the sequence CATCGATG. An *E. coli* transformant harboring
a plasmid having a new Cla I site at the position of the

25   previous EcoR V site was identified, and the plasmid was
named pOO#1 NcoÆ RV>Cla. This DNA was then digested with
Cla I and Nco I, and the small (268 bp) fragment was
purified from an agarose gel. This fragment was then
ligated to the 3429 bp Cla I/Nco I fragment of pUC13/35S(-

30   343) prepared above, and an *E. coli* transformant that
harbored a plasmid having Cla I/Nco I fragments 3429 and
268 bp was identified. This plasmid was named pUC13/35S
En.

The plasmid pUC13/35S En DNA was digested with Nco I, and the protruding ends were made blunt by treatment with T4 DNA polymerase. The treated DNA was then cut with Sma I, and was ligated to Bgl II linkers having the sequence

5   CAGATCTG. An *E. coli* transformant that harbored a plasmid in which the 416 bp Sma I/NcoI fragment had been replaced with at least two copies of the Bgl II linkers was identified, and named p35S En^2. The DNA structure of p35S En^2 was as follows: Beginning with the nucleotide that

10   follows the third C residue of the Sma I site on the strand contiguous to the noncoding strand of the *lacZ* gene of pUC13; the linker sequence CAGATCTGCA GATCTGCATG GGCGATG, followed by CaMV nucleotides 7090 to 7344, followed by the Cla I linker sequence CATCGATG, followed

15   by CaMV nucleotides 7089 to 7443, followed by the Hind III linker sequence CAAGCTT, followed by the rest of pUC13 sequence. This structure had the feature that the enhancer sequences of the CaMV 35S promoter, which laid in the region upstream of the EcoR V site in the viral genome

20   (bases 7090 to 7344), had been duplicated. This promoter construct incorporated the native 35S transcription start site, which was 11 nucleotides upstream of the first A residue of the Hind III site.

     For plasmids utilizing the 35S promoter and the

25   *Agrobacterium* NOS Poly A sequences, the starting material for the first construct was plasmid pBI221, purchased from CLONTECH (Palo Alto, CA). This plasmid contained a slightly modified copy of the CaMV 35S promoter. Beginning at the 3' end of the Pst I site of pUC19

30   (Yanisch-Perron et al., 1985 Gene 33:103-119), and reading on the same strand as that which encodes the *lacZ* gene of pUC19, the sequence was comprised of the linker nucleotides GTCCCC, followed by CaMV nucleotides 6605 to

7439 followed by the linker sequence GGGGACTCTA GAGGATCCCC GGGTGGTCAG TCCCTT. These bases were then followed by 1809 bp comprising the coding sequence of the *E. coli uidA* gene, which encodes the ß-glucuronidase (GUS) protein, and

5 55 bp of 3' flanking bases that are derived from the *E. coli* genome (Jefferson, 1986 Proc. Natl. Acad. Sci. 83:8447-8451), followed by the Sac I linker sequence GAGCTC, which was then followed by the linker sequence GAATTCCCC. These bases were followed by the RNA

10 transcription termination/polyadenylation signal sequences derived from the *Agrobacterium tumefaciens* nopaline synthase (NOS) gene, and comprised the 256 bp Sau3A I fragment corresponding to nucleotides 1298 to 1554 of DePicker *et al.*(1982 J. Molec. Appl. Genet. 1:561-573),

15 followed by two C residues, the Eco RI recognition sequence GAATTC, and the rest of pUC19.

The plasmid pBI221 DNA was digested with EcoR I and BamH I, and the 3507 bp fragment was purified from an agarose gel. Plasmid pRAJ275 (CLONTECH) DNA was digested

20 with EcoR I and Sal I, and the 1862 bp fragment was purified from an agarose gel. These two fragments were mixed together, and complementary synthetic oligonucleotides having the sequence GATCCGGATC CG and TCGACGGATC CG were added. The fragments were ligated

25 together, and an *E.coli* transformant harboring a plasmid having the appropriate DNA structure was identified by restriction enzyme analysis. DNA of this plasmid, named pKA881, was digested with Bal I and Eco RI, and the 4148 bp fragment was isolated from an agarose gel. DNA of

30 pBI221 was similarly digested, and the 1517 bp Eco RI/Bal I fragment was gel purified and ligated to the above pKA881 fragment, to generate plasmid pKA882. Then pKA882 DNA was digested with Sac I, the protruding ends were made

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blunt by treatment with T4 DNA polymerase, and the
fragment was ligated to synthetic BamH I linkers having
the sequence CGGATCCG. An *E.coli* transformant that
harbored a plasmid having BamH I fragments of 3784 and
5   1885 bp was identified and named pKA882B. Following,
pKA882B DNA was digested with BamH I, and the mixture of
fragments was ligated. An *E.coli* transformant that
harbored a plasmid that generated a single 3783 bp
fragment upon digestion with BamH I was identified and
10   named p35S/NOS. This plasmid had the essential DNA
structure of pBI221, except that the coding sequences of
the GUS gene had been deleted. Therefore, CaMV
nucleotides 6605 to 7439 were followed by the linker
sequence GGGGACTCTA GAGGATCCCG AATTTCCCC. The linker
15   sequence was then followed by the NOS Polyadenylation
sequences and the rest of pBI221.

The plasmid p35S/NOS DNA was digested with EcoR V and
Pst I, and the 3037 bp fragment was purified and ligated
to the 534 bp fragment obtained from digestion of p35S En^2
20   DNA with EcoR V and Pst I. An *E. coli* transformant was
identified that harbored a plasmid that generated
fragments of 3031 and 534 bp upon digestion with EcoR V
and Pst I, and the plasmid was named p35S En^2/NOS. This
plasmid contained the duplicated 35S promoter enhancer
25   region described for p35S En^2, the promoter sequences
being separated from the NOS polyadenylation sequences by
linker sequences that included unique Xba I and BamH I
sites.

The MSV genomic sequence was published by Mullineaux
30   *et al.*, (1984 EMBO J. 3:3063-3068), and Howell (1984
Nucleic Acids Res. 12:7459-7375), and the transcript was
described by Fenoll *et al.* (1988 EMBO J. 7:1539-1596).

The entire sequence, comprised 154 bp and was constructed in three stages by assembling blocks of synthetic oligonucleotides. First, complementary oligonucleotides having the sequence GATCCAGCTG AAGGCTCGAC AAGGCAGATC

5   CACGGAGGAG CTGATATTTG GTGGACA and AGCTTGTCCA CCAAATATCA GCTCCTCCGT GGATCTGCCT TGTCGAGCCT TCAGCTG were synthesized and purified by standard procedures. Annealing of these nucleotides into double-stranded structures left 4-base single stranded protruding ends compatible with those

10   generated by BamH I on one end of the molecule (GATC), and with Hind III-generated single stranded ends on the other end of the molecule (AGCT). Such annealed molecules were ligated into plasmid pBluescript SK(-) [hereinafter called pBSK; Stratagene Cloning Systems, La Jolla, CA], that had

15   been digested with BamH I and Hind III. The sequence of these oligonucleotides was such that when ligated onto the respective BamH I and Hind III sticky ends, the sequences of the respective recognition sites were maintained. An *E. coli* transformant harboring a plasmid containing the

20   oligonucleotide sequence was identified by restriction enzyme analysis, and the plasmid was named pMSV A.

Complementary oligonucleotides having the sequences AGCTGTGGAT AGGAGCAACC CTATCCCTAA TATACCAGCA CCACCAAGTC AGGGCAATCC CGGG and TCGACCCGGG ATTGCCCTGA CTTGGTGGTG

25   CTGGTATATT AGGGATAGGG TTGCTCCTAT CCAC were synthesized and purified by standard procedures. Annealing of these nucleotides into double-stranded structures left 4-base sticky ends that were compatible with those generated by Hind III on one end of the molecule (AGCT), and with Sal

30   I-generated sticky ends on the other end of the molecule (TCGA). The sequence of these oligonucleotides was such that when ligated onto the Hind III sticky ends the recognition sequence for Hind III was destroyed.

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DNA of pMSV A was digested with Hind III and Sal I, and was ligated to the above annealed oligonucleotides. An *E. coli* transformant harboring a plasmid containing the new oligonucleotides was identified by restricition enzyme
5   site mapping, and was named pMSV AB.

Complementary oligonucleotides having the sequences CCGGGCCATT TGTTCCAGGC ACGGGATAAG CATTCAGCCA TGGGATATCA AGCTTGGATC CC and TCGAGGGATC CAAGCTTGAT ATCCCATGGC TGAATGCTTA TCCCGTGCCT GGAACAAATG GC were synthesized and
10 purified by standard procedures. These oligonucleotides incorporate bases that comprise recognition sites for Nco I, EcoR V, Hind III, and BamH I. Annealing of these nucleotides into double-stranded structures left 4-base sticky ends that were compatible with those generated by
15 Xma I on one end of the molecule (CCGG), and with Xho I-generated sticky ends on the other end of the molecule (TCGA). Such annealed molecules were ligated into pMSV AB DNA that had been digested with Xma I and Xho I. An *E. coli* transformant harboring a plasmid containing the
20 oligonucleotide sequence was identified by restriction enzyme analysis, and DNA structure was verified by sequence analysis. The plasmid was named pMSV CPL. Together, these comprised the 5' untranslated leader sequence ("L") of the MSV coat protein ("CP") gene. These
25 corresponded to nucleotides 167 to 186, and 188 to 317 of the MSV sequence of Mullineaux *et al.*, (1984), and were flanked on the 5' end by the BamH I linker sequence GGATCCAG, and on the 3' end by the linker sequence GATATCAAGC TTGGATCCC. An A residue corresponding to base
30 187 of the wild type MSV sequence was inadvertently deleted during cloning.

The plasmid pMSV CPL DNA was digested at the Sma I site corresponding to base 277 of the MSV genomic

sequence, and the DNA was ligated to Bgl II linkers having the sequence CAGATCTG. An *E. coli* transformant harboring a plasmid having a unique Bgl II site at the position of the former Sma I site was identified and verified by DNA

5   sequence analysis, and the plasmid was named pCPL-Bgl.

The starting material in construction of a deleted version of the maize alcohol dehydrogenase 1 (Adh1) intron 1 is plasmid pVW119, which was obtained from V. Walbot, Stanford University, Stanford, CA. This plasmid contained

10 the DNA sequence of the maize Adh1.S gene, including intron 1, from nucleotides 119 to 672 [numbering of Dennis *et al.* (1984 Nucleic Acids Res. 12:3983-4000)], and was described in Callis *et al.* (1987 Genes and Develop. 1:1183-1200). In pVW119, the sequence following base 672

15 of Dennis *et al.* (1984) was GACGGATCC. The entire intron 1 sequence, with 14 bases of exon 1, and 9 bases of exon 2, was obtained from this plasmid on a 556 bp fragment following digestion with Bcl I and BamH I.

The plasmid pSG3525a(Pst) DNA was digested with BamH

20 I and Bcl I, and the 3430 bp fragment was purified from an agarose gel. DNA of plasmid pVW119 was digested with BamH I and Bcl I, and the gel purified fragment of 546 bp was ligated to the 3430 bp fragment. An *E. coli* transformant was identified that harbored a plasmid that generated

25 fragments of 3430 and 546 upon digestion with BamH I and Bcl I. This plasmid was named pSG AdhA1.

The DNA of pSG AdhA1 was digested with Hind III and with Stu I. The ends were made flush by T4 DNA polymerase treatment and then ligated. An *E. coli* transformant that

30 harbored a plasmid lacking Hind III and Stu I sites was identified and the DNA structure was verified by sequence analysis. The plasmid was named pSG AdhA1Æ. In this construct, 344 bp of DNA had been deleted from the

interior of the intron 1. The loss of these bases did not
affect splicing of this intron. The functional intron
sequences ware obtained on a 213 bp fragment following
digestion with Bcl I and BamH I.

5   DNA of plasmid pCPL-Bgl was digested with Bgl II, and
the linearized DNA was ligated to the 213 bp Bcl I/BamH I
fragment containing the deleted version of the Adh1.S
intron 1 sequences from pSG AdhA1Æ. An *E. coli*
transformant was identified by restriction enzyme site
10 mapping that harbored a plasmid that contained the intron
sequences ligated into the Bgl II site, in the orientation
such that the Bgl II/Bcl I juncture was nearest the 5' end
of the MSV CPL leader sequence, and the Bgl II/ BamH I
juncture was nearest the 3' end of the CPL. This
15 orientation was confirmed by DNA sequence analysis. The
plasmid was named pCPL A1I1Æ. The MSV leader/intron
sequences was obtained from this plasmid by digestion with
BamH I and Nco I, and purification of the 373 bp fragment.

Construction of plant expression vectors based on the
20 enhanced 35S promoter, the MSV CPL, and the deleted
version of the Adh1 intron 1 was as follows. DNA of
plasmid p35S En^2/NOS was digested with BamH I, and the
3562 bp linear fragment was ligated to a 171 bp fragment
prepared from pMSV CPL DNA digested with BamH I. This
25 fragment contained the entire MSV CPL sequence. An *E.
coli* transformant was identified by restriction enzyme
site mapping that harbored a plasmid that contained these
sequences in an orientation such that the Nco I site was
positioned near the NOS Poly A sequences. This plasmid
30 was named p35S En^2 CPL/NOS. It contained the enhanced
version of the 35S promoter directly contiguous to the MSV
leader sequences such that the derived transcript included
the MSV sequences in its 5' untranslated portion.

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The DNA of plasmid pKA882 was digested with Hind III and Nco I, and the large 4778 bp fragment was ligated to an 802 bp Hind III/Nco I fragment containing the enhanced 35S promoter sequences and MSV leader sequences from p35S

5   En^2 CPL/NOS. An *E. coli* transformant harboring a plasmid that contained fragments of 4778 and 802 bp following digestion with Hind III and Nco I was identified, and named pDAB310. In this plasmid, the enhanced version of the 35S promoter was used to control expression of the GUS

10 gene. The 5' untranslated leader portion of the transcript contains the leader sequence of the MSV coat protein gene.

DNA of plasmid pDAB310 was digested with Nco I and Sac I. The large 3717 bp fragment was purified from an

15 agarose gel and ligated to complementary synthetic oligonucleotides having the sequences CGGTACCTCGAGTTAAC and CATGGTTAACTCGAGGTACCGAGCT. These oligonucleotides, when annealed into double stranded structures, generated molecules having sticky ends compatible with those left by

20 Sac I, on one end of the molecule, and with Nco I on the other end of the molecule. In addition to restoring the sequences of the recognition sites for these two enzymes, new sites were formed for the enzymes Kpn I (GGTACC), Xho I (CTCGAG), and Hpa I (GTTAAC). An *E. coli* transformant

25 was identified that harbored a plasmid that contained sites for these enzymes, and the DNA structure was verified by sequence analysis. This plasmid was named pDAB1148.

DNA of plasmid pDAB1148 was digested with Bam HI and

30 Nco I, the large 3577 bp fragment was purified from an agarose gel and ligated to a 373 bp fragment purified from pCPL A111Æ following digestion with Bam HI and Nco I. An *E. coli* transformant was identified that harbored a

plasmid that generated fragments of 3577 and 373 bp
following digestion with BamH I and Nco I, and the plasmid
was named pDAB303. This plasmid has the following DNA
structure: beginning with the base after the final G
5 residue of the Pst I site of pUC19 (base 435), and reading
on the strand contiguous to the coding strand of the lacZ
gene, the linker sequence ATCTGCATGG GTG, nucleotides 7093
to 7344 of CaMV DNA, the linker sequence CATCGATG,
nucleotides 7093 to 7439 of CaMV, the linker sequence
10 GGGGACTCTA GAGGATCCAG, nucleotides 167 to 186 of MSV,
nucleotides 188 to 277 of MSV, a C residue followed by
nucleotides 119 to 209 of Adh1.S, nucleotides 555 to 672
of maize Adh1.S, the linker sequence GACGGATCTG,
nucleotides 278 to 317 of MSV, the polylinker sequence
15 GTTAACTCGA GGTACCGAGC TCGAATTTCC CC containing recognition
sites for Hpa I, Xho I, Kpn I, and Sac I, nucleotides 1298
to 1554 of NOS, and a G residue followed by the rest of
the pUC19 sequence (including the EcoR I site).

     DNA of plasmid pDAB303 was digested with Nco I and
20 Sac I, and the 3939 bp fragment was ligated to the 1866 bp
fragment containing the GUS coding region prepared from
similarly digested DNA of pKA882. The appropriate plasmid
was identified by restriction enzyme site mapping, and was
named pDAB305. This plasmid had the enhanced promoter,
25 MSV leader and Adh1 intron arrangement of pDAB303,
positioned to control expression of the GUS gene.

     The plasmid pDAB305 contained an enhanced 35S
promoter with additional 3' sequences and embodied as
nucleotides 7093 to 7344 of CaMV DNA, the linker sequence
30 CATCGATG, nucleotides 7093 to 7439 of CaMV, the linker
sequence GGGGACTCTA GAGGATCCAG, nucleotides 167 to 186 of
MSV, nucleotides 188 to 277 of MSV, a C residue followed
by nucleotides 120 to 210 of maize Adh1.S, nucleotides 555

to 672 of maize Adh1.S, the linker sequence GACGGATCTG,
nucleotides 278 to 317 of MSV, and a G residue that
represents the final base of an Nco I recognition
sequence, CCATGG. As above, the GUS translational start
5   codon was part of the Nco I site. Transcripts from this
promoter contain as the 5' untranslated leader essentially
the MSV coat protein leader sequence, into which has been
inserted a deleted version of the maize Adh1.S intron 1.

10

<u>Claims</u>

1.   A method for transforming a plant cell which comprises:

      (a)   selecting at least one cell from a Type I, Type
5   II, hypocotyl-derived, or cotyledon-derived callus culture
or from a meristem, pollen, cotyledon, or germ cell
tissue; and

      (b)   inserting DNA into said cell by whisker mediated
transformation.

10   2.   A method for producing a fertile transgenic plant
which comprises regenerating a cell produced by the method
of claim 1.

3.   A method for producing a fertile transgenic plant
comprising the steps of: (i) establishing a regenerable
15   callus culture from a plant to be transformed wherein said
callus culture is selected from the group consisting of
Type I, Type II, hypocotyl-derived, and cotyledon-derived
callus culture; (ii) selecting a plant cell aggregate
therefrom for transformation; (iii) transforming said
20   plant cell aggregate with DNA by whisker-mediated
transformation; (iv) identifying transformed cell lines
therefrom; and (iv) regenerating fertile transgenic plants
therefrom.

4.   The method of Claim 3 wherein said Type I callus
25   culture is established from *Zea mays* or *Oryza sativa*.

5.   The method of Claim 3 wherein said Type II callus
culture is established from *Zea mays*.

6.   The method of Claim 3 wherein said hypocotyl-derived
culture is established from *Gossypium hirsutum*.

30   7.   The method of Claim 3 wherein said cotyledon-derived
culture is established from *Gossypium hirsutum*.

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8. The method of Claim 3 wherein said plant cell aggregate is initiated on solid medium.

9. A fertile transgenic plant produced by the method of any one of claims 3-8.

5 10. A DNA construct functional in a plant cell comprising in the 5' to 3' direction of transcription, a transcriptional regulatory region functional in said plant cell and having a DNA sequence according to SEQ ID NO:1, and a gene of interest, said gene being either in the 10 sense or antisense orientation.

11. The transcriptional regulatory region according to Claim 10 comprising the DNA sequence SEQ ID NO:1.

SEQUENCE LISTING

```
<110> Petolino, Joseph F
      Pareddy, Dayakar R
      Hopkins, Nicole L
      Armstrong, Katherine

<120> Whisker Mediated Transformation of Plant Cell
      Aggregates and Plant Tissues and Regeneration of Plants

<130> 50518

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atctcgatac accaaatcga ctctagaact agtggatccg tcgacc          706
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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   C12N15/31   C12N15/82   A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C12N   A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 302 523 A (COFFEE RONALD ET AL) 12 April 1994 cited in the application see the whole document ---	1
X	WO 94 28148 A (ZENECA LTD) 8 December 1994 cited in the application see the whole document ---	1,2
X	NAGATANI N. ET AL.: "DNA delivery into rice cells and transformation using silicon carbide whiskers" BIOTECHNOLOGY TECHNIQUES, vol. 11, no. 7, 1997, pages 471-473, XP002103973 see the whole document ---	1,2

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[X] Further documents are listed in the continuation of box C   [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 May 1999	10/06/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kania, T

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No
A	WO 92 09696 A (PLANT GENETIC SYSTEMS NV) 11 June 1992 see the whole document ---	1-5,8,9
A	WO 97 12512 A (CALGENE INC) 10 April 1997 ---	1-3,6-9
A	WANG K. ET AL.: "Whisker-mediated plant transformation: an alternative technology" IN VITRO CELLULAR AND DEVELOPMENTAL BIOLOGY, vol. 31, 1995, pages 101-104, XP002103974 see the whole document ---	1-9
A	SONGSTAD D. ET AL.: "Advances in alternative DNA delivery techniques" PLANT CELL,TISSUE AND ORGAN CULTURE, vol. 40, 1995, pages 1-15, XP002103975 see p.5 2.par - p.6 3.par. ---	1-9
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INTERNATIONAL SEARCH REPORT

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